Article

Polyketide Synthase (PKS)-containing foliar endophytes from a traditional rice variety exhibit antagonism against *Xanthomonas oryzae* pv. *oryzae*

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Abstract

Rice (*Oryza sativa* L.), a staple diet for the majority of Asians and considered one of the most crucial food crops, often suffer from diseases like Bacterial Leaf Blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae*. Endophytic fungi harbor secondary metabolites, including polyketides, which exhibit potent medicinal properties and, importantly, possess agricultural potential. In this study, we investigated the capability of ethyl acetate extracts derived from foliar endophytic fungi present in Diket red rice to produce bioactive antibacterial compounds. The extracts were tested for their *in vitro* antimicrobial activity against *X. oryzae* pv. *oryzae*, and the presence of polyketide synthase (PKS) genes was also examined. Among the tested isolates, namely *Aspergillus spelaeus*, *Penicillium steckii*, *Penicillium chrysogenum*, and *Cladosporium oxysporum*, the latter exhibited the most statistically ($p>0.05$) significant reduction in optical density (OD 600) of *X. oryzae* pv. *oryzae* broth cultures compared to positive control (Chloramphenicol), indicating possible antimicrobial activity. Out of the 16 fungal isolates, 15 were found to have the PKS gene, which may account for their antagonistic potential. These findings highlight *A. spelaeus*, *P. steckii*, *P. chrysogenum*, and *C. oxysporum* as promising candidates for the isolation of bioactive compounds aimed at controlling BLB in the future.

Keywords endophytic fungi; Diket red rice; polyketide; *Xanthomonas oryzae* p.v. *oryzae*.

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1 Introduction

Rice (*Oryza sativa* L.) plays a crucial role in global agriculture as one of the most important crops, providing sustenance to more than half of the world's population (Khush, 2005). It serves as a staple diet for the majority

of Asians and holds significant importance as a primary food crop. However, rice cultivation is constantly challenged by phytopathogens, leading to various rice-related diseases.

According to the International Rice Research Institute, *Xanthomonas oryzae* pv. *oryzae*, the causative agent of bacterial leaf blight (BLB), poses a significant threat and has historically been associated with devastating epidemics in Asia. Farmers, on average, experience yield losses of 37% due to pests and diseases, with the magnitude ranging from 24% to 41% depending on specific production conditions (International Rice Research Institute, 2003). When susceptible rice varieties are affected by bacterial blight, yield losses can escalate to as high as 70% (Vera Cruz, et al., 2015). In light of current agricultural trends, there is a growing emphasis on reducing the use of chemical pesticides and exploring alternative approaches that enhance environmental quality (Kusari et al., 2013).

As the global population continues to rise, projected to reach 9.7 billion by 2050 (Ray et al., 2019), ensuring stable rice production is paramount. This growing demand further highlights the urgency of addressing challenges like BLB and exploring sustainable disease management strategies.

The utilization of plant-microorganism interactions, particularly rice-endophytic fungi mutualisms, holds promise as an alternative approach. Fungal secondary metabolites, known for their effectiveness in drug development, are of significant interest due to their agricultural potential (Newman and Cragg, 2012). Studies have shown that endophytic fungi can produce various bioactive compounds, including polyketides with antibacterial properties against rice pathogens like *Magnaporthe oryzae* and *Fusarium fujikuroi* (Kumar et al., 2020; Liu et al., 2023). Interestingly, research by Wang et al. (2020) demonstrated that endophytic *Penicillium oxalicum* isolated from *Stellera chamaejasme* L. produced polyketides exhibiting potent antibacterial activity against *Xanthomonas oryzae* pv. *oryzae*, the very pathogen causing BLB. This finding highlights the potential of endophytic fungi as natural biocontrol agents for managing this critical rice disease.

Endophytic fungi reside within the internal cavities of plant tissues, including roots, stems, and leaves, and typically emerge to sporulate during plant or host-tissue senescence (Rodriguez et al., 2009). According to Hipol (2014), it is widely accepted that fungal endophytes colonize living plant tissues without causing immediate negative effects. In fact, certain endophytic fungi confer benefits to their hosts, such as enhanced drought tolerance, protection against pathogens, improved growth, and defense against herbivory, thereby contributing to the host's fitness (Higginbotham et al., 2013).

Polyketides have been a focal point of extensive research (Hopwood, 1997). They are a class of secondary metabolites produced by various organisms, with bacteria and fungi being the most extensively studied (Amnuaykanjanasin et al., 2005). Considered a valuable group in the realm of therapeutic drugs, polyketides have been recognized as a rich "drug gold mine" (Borchardt, 1999). Fungal polyketides encompass a range of compounds, including mycotoxins like aurofusarin (Shibata et al., 1966), aflatoxin (Bhatnagar et al., 2003), and zearalenone (Urry et al., 1966), as well as spore pigments (Mayorga and Timberlake., 1992). Genes known as polyketide synthase (PKS) genes encode these polyketides. For instance, certain actinomycetes synthesize two chemically distinct metabolites encoded by separate PKS genes, both targeting the same organism. This phenomenon suggests that unrelated compounds may be selected for the same activity when vital for the organism's survival (Challis, 2003). The presence of PKS genes in fungi implies their potential in producing intriguing and promising natural products.

Numerous studies have been dedicated to the characterization of germplasm and DNA fingerprinting of traditional rice varieties (TRVs). These varieties possess exceptional traits, such as high resistance to insects and pathogens (Tad-awan et al., 2010), making them ideal host plants for the isolation of endophytic fungi. Among these intriguing TRVs, Diket red rice cultivated in Bokod, Benguet, stands out as a particularly

interesting variety. Notably, studies suggest Diket red rice exhibits resistance to various diseases, including reports of BLB resistance (Siam et al., 2016). Further research into the specific resistance mechanisms of Diket red rice to BLB could provide valuable insights for future breeding programs and strengthen its suitability for harboring potentially beneficial endophytic communities.

Therefore, the primary objective of this study was to identify foliar endophytic fungi obtained from Diket red rice and investigate the presence of polyketide synthase (PKS) genes of the isolated endophytes. The specific objectives were as follows: (1) identify the endophytic fungi using ITS primers; (2) determine the presence of PKS genes in the isolated endophytic fungi; (3) conduct phylogenetic analysis using the ITS primers and the deduced amino acid sequences derived from the PKS gene sequences; (4) assess the biocontrol activity of the endophytic fungi through *in vitro* antagonistic activity against *Xanthomonas oryzae* pv. *oryzae*.

2 Materials and Methods

2.1 Isolation and morphological characterization of endophytic fungi

The isolation of endophytic fungi was carried out using the leaves of the traditional Cordillera rice cultivar, Diket red rice (Fig. 1), in ripening period (milky stage). Three sites, in one (1) rice field location were selected. Per site, three individual rice plants were sampled, and 3 leaves were collected in each sample. Only visually healthy leaves were chosen for the isolation process. The plant materials were thoroughly washed with running water, and the selected healthy leaves were then cut into smaller pieces measuring 3 - 5 cm in length under sterile conditions. To ensure sterility, the leaves underwent surface sterilization by being treated with 95% ethanol for 1 minute, followed by 10% NaOCl for 5 minutes (Wang et al., 2016). After the sterilization process, the leaf segments were rinsed three times with sterile distilled water and air-dried for 20 minutes inside a laminar hood. The effectiveness of the surface sterilization was assessed by plating 100 µL of the final distilled water rinse on potato dextrose agar (PDA) plates.

Fig. 1 Photo showing the Diket red rice panicles and leaves.

Endophytic fungi were isolated by placing the sterilized leaf segments onto PDA plates supplemented with 150 ppm chloramphenicol to prevent bacterial growth and 200 ppm Rose Bengal to inhibit the growth of fast-growing fungi. The Petri dishes containing the plated leaf segments were incubated in darkness at 28°C and monitored daily to observe the growth of endophytic fungal hyphae emerging from the segments. Individual

hyphal tips from various fungi were aseptically removed from the agar plates and transferred to new plates, which were then incubated at 28°C for a minimum of 10 days. Each fungal culture was checked for purity and subsequently transferred to another PDA plate using the hyphal tip method (Strobel et al., 1996). To ensure that surface sterilization had removed all hyphae and chlamydospores externally adhering to the segments, the surface-sterilized segments were imprinted simultaneously in PDA agar plates and incubated under the same conditions (Kusari et al. 2013). Only segments that were negative in this test were used for endophyte isolation. The fungal isolates were temporarily labeled and stored on the surface of PDA slants at 4°C.

By observing the morphological and cultural characteristics of colonies on plates, all fungal isolates were tentatively grouped. Colony morphology such as form, color, texture, margin and surface elevation were observed using stereomicroscope while the cell morphology was documented using an Optika B-159R compound light following the slide culture technique (Riddle, 1950) with lactophenol-methyl blue as stain. EC-300 camera mount was used for capturing the image of the mycelial body and spores of the endophytic fungi. Morphologically distinct fungal isolates were subjected to molecular identification methods.

2.2 Genomic DNA extraction and molecular identification

Genomic DNA extraction for PCR amplification was conducted using the cetyltrimethylammonium bromide (CTAB) method, as described by Liu et al. (2012) and Pablo et al. (2020). A fresh lump of fungal mycelia obtained from PDA cultures was placed in a 1.5 mL Eppendorf tube along with 500 µL of lysis buffer (400 nM Tris-HCl [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaCl, 1% sodium dodecyl sulfate). The mycelia were disrupted using a sterile inoculating needle, and the tube was briefly vortexed after the addition of 150 µL of potassium acetate. Subsequently, the tube was centrifuged at 10,000 xg for 5-10 minutes at 4°C.

To precipitate the DNA, 500 µL of the supernatant was transferred to another 1.5 mL Eppendorf tube, and 30 µL of sodium acetate and 1 mL of cold ethanol were added. The tube was inverted several times after each solution addition. After placing the tube in the freezer for 20 minutes, it was centrifuged at 12,500 xg for 15 minutes, and the supernatant was discarded. The remaining DNA pellet was mixed with 1 mL of 70% ethanol and briefly spun to decant the supernatant. The DNA pellet was then air-dried overnight, resuspended in TE buffer, and stored at -20°C for further use.

The identification of fungal isolates were done through the internal transcribed region (ITS) of the 18S rDNA via the universal primers ITS-1 (5'-TCC GTA GGT GAA CCT GCG G- 3') as a forward primer and ITS -4 (5'-TCC TCC GTC TAT TGA TAT GC -3') as the reverse primer. The PCR cocktail (50 µL) contained 25 µL of the Emerald-AMP PCR Master Mix (Takara), 1 µL each of the forward and reverse primers, 23 µL of PCR water, and 1 µL of the gDNA. The conditions of the reaction cycle consisted of 5 min initial denaturation at 95°C, 35 cycles of denaturation at 95°C for 30s, annealing at 60°C for 1 min, extension at 72°C for 1 min and a final extension phase of 6 min at 72°C. To check the generation of amplicons with the desired length of about 550 bp, the PCR products were run on 1% agarose gel. The PCR products were sent to the 1st Base Sequencing Facility in Singapore for cleaning and subsequent sequencing using ITS-1 primer.

For strain identification, comparison of the sequence homology of the nucleotide of the 18S rDNA ITS region of the fungi were accomplished via the Basic Local Alignment Sequence Tools using nucleotides (BLASTn) search program (http://www.ncbi.nlm.nih.gov/BLAST). Similarity greater than 97% within the isolates was considered to belong in the same species. Among the endophytic species, the 3% difference was used to define species boundaries that correlate well within their differences (Arnold and Lutzoni, 2007). Genus were accepted within the range of 95-97% similarity. If the sequence similarity is below 95%, the isolate was treated as unidentified (Sanchez-Marquez et al, 2008; Aban, 2019). Verification and confirmation

of the results from BLASTn search was strengthened via the colony morphology and microscopic examinations.

The Molecular Evolutionary Genetics Analysis software (MEGA) was used for both strain identification using 18S rDNA ITS region sequences to construct phylogenetic analysis of the sequences of the identified endophytes and the closely related species (Tamura et al., 2013). The sequences were aligned using ClustalW-Pairwise Sequence Alignment and Maximum Likelihood (ML) tree with 1,000 bootstrap replications to reveal the genetic distances between the different fungal strains intuitively and to obtain statistical support. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

2.3 PKS gene screening and phylogenetic analysis

PKS genes were amplified from the genomic DNA of the identified fungal isolates using degenerate primers designed for the highly conserved sequences of ß-ketoacyl synthase (KS) domains, which are shared among all PKSs. The primer pair KAF1/KAR1 (5'-GAR KSI CAY GGI ACI GGI AC-3'/5'-CCA YTG IGC ICC RTG ICC IGA RAA-3') as described by Amnuaykanjanasin et al. (2015) was employed to detect PKS genes in the fungal isolates. The PCR reaction was carried out in a 50 µL reaction mixture, consisting of 25 µL of 2x Taq PCR Mastermix, 2 µL of the forward primer (10 µM), 2 µL of the reverse primer (10 µM), 2 µL of template DNA, and 19 µL of sterile double-distilled water. The PCR conditions followed the protocol outlined by Wang et al. (2015): an initial denaturation step at 95°C for 4 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 1 minute, extension at 72°C for 2 minutes, and a final extension step at 72°C for 10 minutes.

The obtained PKS gene sequences were subjected to analysis using BLASTx to compare and align them against reference sequences. The alignment was performed using CLUSTAL X (Thompson et al., 1997). The nucleotide sequences were translated into protein sequences using the ORF FINDER tool available on the NCBI website (http://www.ncbi.nlm.nih.gov/projects/gorf/). The deduced amino acid sequences derived from the nucleotide sequences were then used as queries in a BLASTp search against the nr protein database to identify related proteins. To infer the evolutionary relationships, an unrooted phylogenetic tree based on the amino acid sequences of the KS domain was constructed. The neighbor-joining method implemented in MEGA 6.0 was used for tree construction, and bootstrap analysis with 1,000 replicates was performed to assess the robustness of the tree topology.

2.4 *In vitro* antagonistic activity of endophytic fungi against *X. oryzae* pv. *oryzae*

The fungal isolates were cultured in 300 mL of PDA broth and incubated in a shaking incubator at 28°C and 120 rpm for a period of 10 to 15 days. To obtain a homogenized mixture, the broth-mycelial mixture was blended for 5-10 minutes using a blender. Subsequently, the homogenized mixture was filtered to separate the mycelial-free broth. For the extraction of bioactive compounds, the Separatory Funnel Extraction Procedure (Zubrick, 2001) was employed. A 1:3 ratio of broth to ethyl acetate was poured into a separatory funnel and vigorously shaken for 10 minutes. The organic phase, containing the extracted compounds, was collected and transferred to a rotary evaporator. The sample was evaporated under reduced pressure for approximately 45 minutes. The remaining sample was then carefully transferred to a pre-weighed petri plate and left to air-dry for 24 hours. The final weight of the petri plate with the dried sample was recorded prior to the suspension of the sample in Dimethyl sulfoxide (DMSO).

The obtained weight of the dried sample was used to prepare a concentration of 20 mg/mL in Dimethyl sulfoxide (DMSO). This concentration was utilized for the antagonistic activity assay against *X. oryzae* pv. *oryzae* using a modified OD-600 Bacterial Assay, as described by Matlock et al. (2011). The assay was performed in triplicates, with the antibiotic chloramphenicol serving as a positive control. Negative controls

consisted of distilled water and DMSO. The *X. oryzae* pv. *oryzae* pathogen was obtained from the Philippine Rice Research Institute-Isabela. The bacterial samples were cultured in nutrient broth and incubated for 16 hours following the protocol outlined by Zhao et al. (2012). The ethyl acetate extract suspensions, as well as distilled water (negative control), were added to each *X. oryzae* pv. *oryzae* broth in test tubes to achieve a final concentration of 2.00 mg/mL.

The prepared cultures were read three times for each replicate using a Shimadzu UV Visible Spectrophotometer model UV mini-1240 at a wavelength of 600 nm. The initial absorbance was recorded, and after 24 hours, the samples were read again to determine the final absorbance. The changes in absorbance was determined by getting the difference in final and initial absorbance:

$$\Delta OD = OD600_{t=24\,h} - OD600_{t=0\,h} \quad (1)$$

2.5 Data analysis

The collected data from all sixteen (16) isolates, and the positive (chloramphenicol) and negative control (DMSO, and sterile distilled water) were subjected to Analysis of Variance (ANOVA) with a significance level of $p>0.05$, followed by a post-hoc test using the Least Significant Difference (LSD) method. The average of triplicate was used for statistical analysis.

3 Results and Discussion

3.1 Isolation and morphological characterization of endophytic fungi

Sixteen colonies were isolated from nine individuals of Diket red rice (*O. sativa*) , with three leaf samples, per individual. Table 1 summarized the colony characteristics of endophytic fungi. Out of 16, twelve (12) colony morphospecies were initially identified to be morphologically and microscopically distinct, and thus, these were extracted with genomic DNA. Fig. 2 shows the representative genus *Aspergillus* sp., *Penicillium* sp., and *Cladosporium* sp.

Table 1 Colony morphology of the 16 foliar endophytic fungi isolated from Diket Red rice.

ISOLATE CODE	OBVERSE COLOR	REVERSE COLOR	MARGIN	ELEVATION
S1I1	**White**	**Light orange**	**Undulate**	**Raised**
S1I2	**Light cream**	**Cream**	**Filamentous**	**Umbonate**
S1I3	**White**	**White**	**Undulate**	**Raised**
S1I4	**Moss green with white margin**	**Peach**	**Undulate**	**Convex**
S1I5	**White**	**Light orange**	**Undulate**	**Raised**
S2I2	**White**	**Light orange**	**Undulate**	**Raised**
S2I5	**Green with white margin**	**Cream**	**Entire**	**Raised**
S2I7	**White with black spores**	**Cream**	**Filamentous**	**Convex**
S3I1	**Moss green with white margin**	**Peach**	**Undulate**	**Pulvinate**
S3I2	**Moss green with white margin**	**Light orange**	**Undulate**	**Raised**
S3I3	**White**	**Peach**	**Undulate**	**Umbonate**
S3I4	**Moss green with white margin**	**Light orange**	**Undulate**	**Convex**
S3I7	**Moss green with white margin**	**Peach**	**Undulate**	**Convex**
S3I8	**White**	**White**	**Filamentous**	**Flat**
S3I9	**Moss green (center), blue green and white margin**	**Peach**	**Entire**	**Convex**

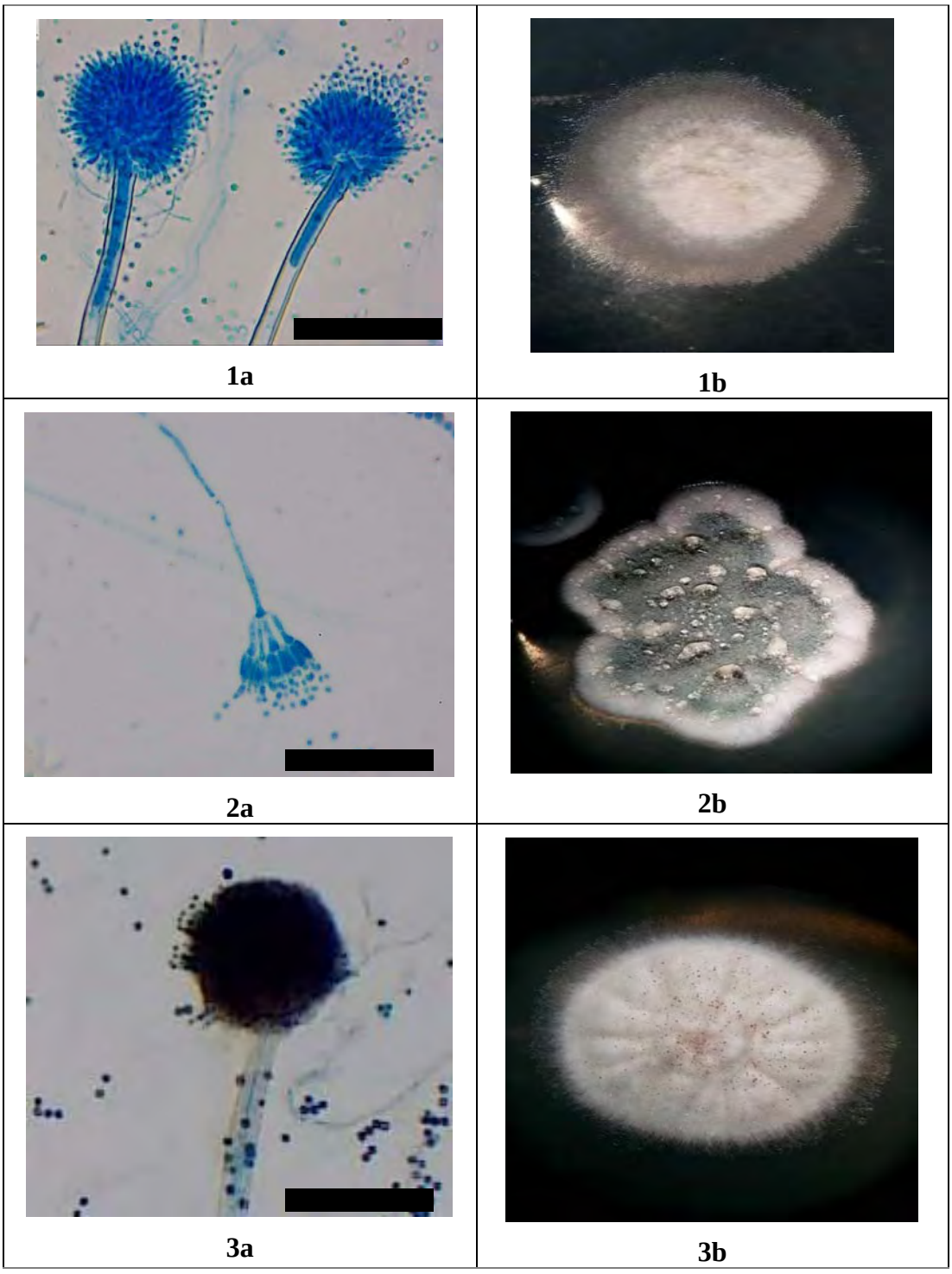

Fig. 2 Microscopic HPO (a) and 24 hr fungal colony morphology (b) of isolates S1I2 (*Aspergillus* sp. – 1a, 1b), SII4 (*Penicillium* sp. – 2a, 2b), and S2I7 (*Cladosporium* sp – 3a, 3b).

3.2 Molecular identification and phylogenetic analysis

Table 2 shows the identified strain through a comparison of the sequence homology of the nucleotide of the 18S rDNA ITS region of the fungi using (BLASTn) search program (http://www.ncbi.nlm.nih.gov/BLAST). Identified closest match of endophytic fungi with 99% identity were: *Aspergillus tubingensis* (NR_131293.1), *Aspergillus spelaeus* (NR_135440.1), *Penicillium chrysogenum* (NR_077145.1), *Penicillium steckii*

(NR_111488.1), *Cladosporium oxysporum* (JX156364.1), and *Penicillium crustosum* (JX869565.1). *Aspergillus tabacinus* (NR_135361.1) had 98% identity similarity.

Table 2 Molecular identification of the fungal endophyte isolates using BLASTn algorithm of NCBI.

Endophytic Fungi Isolate	Closest Match	GenBank Accession #	Total Score	Query Cover (%)	E - Value	Identity (%)
S1I1	***Aspergillus tubingensis***	**NR_131293.1**	**1038**	**94**	**0**	**99**
S1I2	***Aspergillus spelaeus***	**NR_135440.1**	**955**	**93**	**0**	**99**
S1I3	***Aspergillus tubingensis***	**NR_131293.1**	**993**	**96**	**0**	**99**
S1I4	***Penicillium chrysogenum***	**NR_077145.1**	**964**	**96**	**0**	**99**
S1I5	***Aspergillus tubingensis***	**NR_131293.1**	**984**	**97**	**0**	**99**
S2I5	***Aspergillus tubingensis***	**NR_131293.1**	**993**	**97**	**0**	**99**
S3I1	***Penicillium steckii***	**NR_111488.1**	**924**	**89**	**0**	**99**
S3I2	***Cladosporium oxysporum***	**JX156364.1**	**742**	**85**	**0**	**99**
S3I3	***Aspergillus tabacinus***	**NR_135361.1**	**915**	**95**	**0**	**98**
S3I4	***Penicillium chrysogenum***	**NR_077145.1**	**957**	**95**	**0**	**99**
S3I7	***Penicillium chrysogenum***	**NR_077145.1**	**911**	**88**	**0**	**99**
S3I9	***Penicillium crustosum***	**JX869565.1**	**955**	**96**	**0**	**99**

Fig. 3 presents the unrooted ML tree where both *Aspergillus* and *Penicillium* genera clustered while they diverged from genus *Cladosporium*. The genera *Aspergillus* and *Penicillium* fall under Trichocomaceae while genus *Cladosporium* fall under Mycosphaerellaceae.

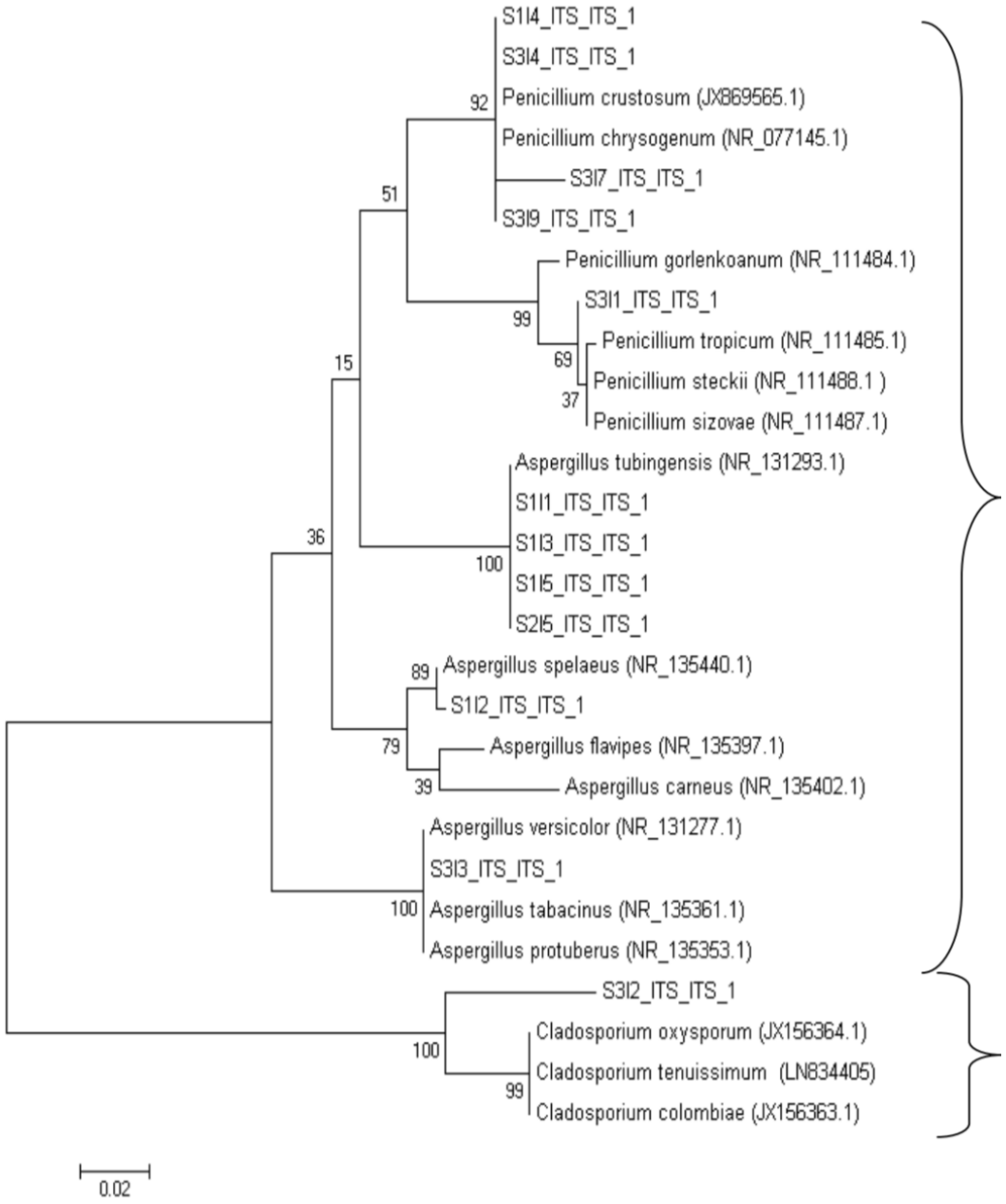

Fig. 3 Unrooted phylogenetic tree of ITS1 of isolates using Maximum Likelihood (ML) method based on the Kimura 2-parameter mode.

3.3 PKS genes screening and phylogenetic analysis

KS domain was detected by KAF1/KAR1 as presented in the agarose gel electrophoresis UV image (Fig. 4). Fifteen isolates were detected with the PKS gene at approximately 750 base pairs. Table 3 shows the closest match of KS domains of fungal endophyte isolates with antimicrobial activity. S1I2 had the closest match (91%) with *Eutypa lata* putative polyketide synthase protein mRNA, S3I1 with *Fusarium gaditjirri* putative polyketide synthase mRNA (89%), S3I2 with *Mycosphaerella graminicola* polyketide synthase (PKS2) mRNA (76%), and S3I4 with *Fusarium sp*. ketoacyl synthase gene (67%).

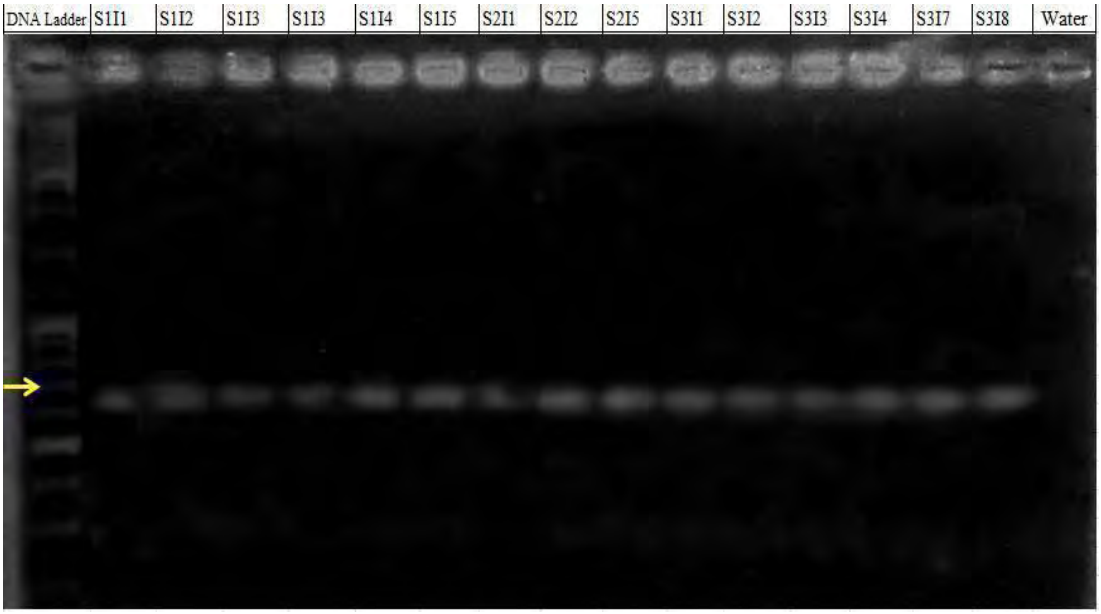

Fig. 4 Agarose gel electrophoresis of the PCR products of ketoacyl domain of PKS gene of 15 endophytic fungi isolates.

Table 3 Closest match of ketoacyl domains of fungal endophyte isolates with antimicrobial activity using NCBI BLASTn algorithm.

Endophytic Fungi Isolate	Closest Match	GenBank Accession #	Total Score	Query Cover (%)	E - Value	Identity (%)
S1I2	***Eutypa lata*** **putative polyketide synthase protein mRNA**	**XM_007796013.1**	**48.2**	**4**	**0.38**	**91**
S3I1	***Fusarium gaditjirri*** **putative polyketide synthase mRNA**	**KU179995.1**	**46.4**	**4**	**0.97**	**89**
S3I2	***Mycosphaerella graminicola*** **polyketide synthase (PKS2) mRNA**	**XM_003849597.1**	**158**	**39**	**1e-34**	**76**
S3I4	***Fusarium sp.*** **ketoacyl synthase gene**	**HQ234258.1**	**62.6**	**28**	**1e-05**	**67**

Fig. 5 shows the unrooted phylogenetic tree of PKS genes using KAF1 primer. The isolates S3I4, S3I1, and S1I2 were grouped under *Penicillium* sp. X13-105 ketoacyl synthase gene (HQ234249.1) while S3I2 was grouped under *Cladosporium* sp. F5 polyketide synthase gene (JQ407022.1).

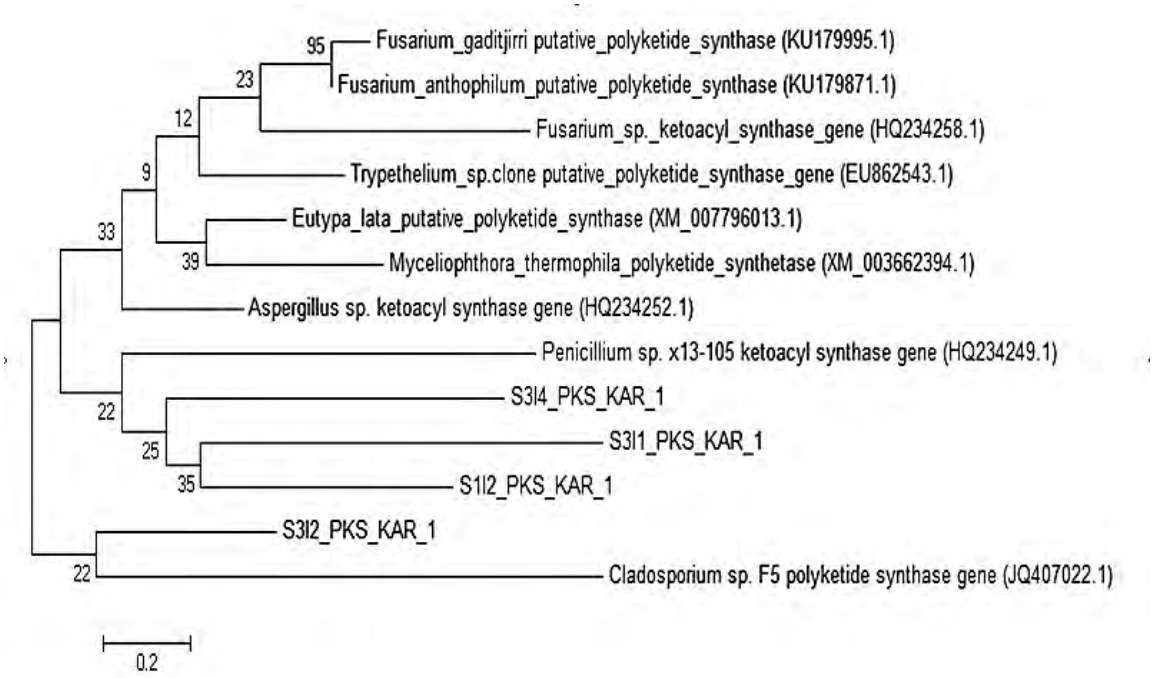

Fig. 5 Unrooted phylogenetic tree of PKS genes using the KAF1 sequencing primer. The tree was constructed through the Maximum Likelihood (ML) method based on the Kimura 2-parameter model.

3.4 Antimicrobial activity using OD 600

The 15 isolates with PKS genes were screened for antimicrobial activity using the OD 600 protocol against the rice phytopathogen, *X. oryzae* pv. *oryzae*. Four (4) isolates showed statistical significance compared to the positive control, chloramphenicol (Fig. 6). These were *Aspergillus spelaeus* (NR_135440.1), *Penicillium steckii* (NR_111488.1), *Penicillium chrysogenum* (NR_077145.1), and *Cladosporium oxysporum* (JX156364.1), and they show possible antagonist against *X. oryzae* pv. *oryzae*. Figure 6 emphasizes that the 4 isolates is statistically comparable to Chloramphenicol (*p>0.5)* using ANOVA, with the change of absorbance values even lower than that of the positive control.

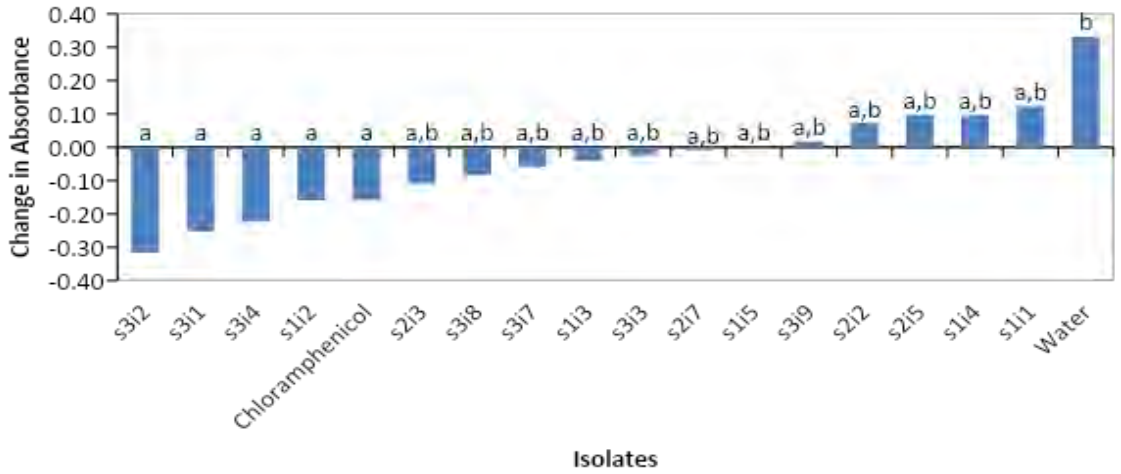

Fig. 6 Antagonistic assay of isolated endophytic fungi using OD600 based on the change in absorbance. *Note: All means of the same letter and designation have no significant difference against each other at 0.05 level of significance using Post hoc test LSD*

4 Discussion

This study focused on isolating and characterizing foliar endophytic fungi from Diket red rice. A total of sixteen strains were obtained and examined based on their colony morphology characteristics (Table 1). Additionally, twelve selected isolates underwent molecular identification using rDNA-ITS sequences to gain a more precise understanding of their genetic composition. The identified endophytic fungal strains were categorized into three genera: *Cladosporium* sp., *Aspergillus* sp., and *Penicillium* sp.

A total of 7 out of 12 identified endophytic strains were distinct species. In the genus *Aspergillus*, three distinct species were authenticated: 99% *Aspergillus tubingensis* (S1I1, S1I3, S1I5, and S2I5), 99% *Aspergillus spelaeus* (S1I2), 98% *Aspergillus tabacinus* (S3I3). In the genus *Penicillium*, also, three distinct species were identified: 99% *Penicillium chrysogenum* (S1I4, S3I4 and S3I7), 99% *Penicillium steckii* (S3I1) and 99% *Penicillium crustosum* (S3I9). While, in genus *Cladosporium*, only one distinct species was authenticated: 99% *Cladosporium oxysporum* (S3I2). Traditionally, *Aspergillus* and *Penicillium* resided within the Trichocomaceae family and Cladosporium belonged to the Mycosphaerellaceae family (Cannon & Kirk, 2007). Currently, both *Aspergillus* and *Penicillium* have been reclassified within the Aspergillaceae family, placed within the order Eurotiales. This family is characterized by well-developed, septate hyphae, conidiophores ranging from unbranched to simply branched, and unicellular conidia produced in chains or clusters. While *Cladosporium* remains in the Cladosporiaceae family. This family is typically recognized by its dematiaceous hyphae, branching conidiophores with sympodial development, and smooth-walled or echinulate conidia produced in chains or clusters. Interestingly, *Cladosporium* species often function as saprobes or plant pathogens, with some species even causing human infections (Taylor et al., 2017)

Interestingly, this study is the first to isolate foliar endophytic fungi from the traditional Cordillera rice variety, Diket red rice. However, the genera *Aspergillus*, *Penicillium*, and *Cladosporium* that were isolated and identified in this study have been frequently associated with rice plants in previous research. Naik et al. (2006) reported the dominance of *Streptomyces* sp., *Chaetomium globosum*, *Penicillium chrysogenum*, *Fusarium oxysporum*, and *Cladosporium cladosporioides* in *Oryza sativa* samples collected from the Bhadra River Project Area in Southern India. Similarly, Wang et al. (2015) identified 19 genera in seven orders of Ascomycota, including unidentified *Pleosporales*, *Phoma*, *Cladosporium*, and *Penicillium*, in their study on culturable endophytic fungi. These findings suggest that certain species of Ascomycota fungi are commonly found in colonizing rice plants. Consistent with previous studies, all the isolated fungi in our study belong to the Phylum Ascomycota. This is in line with the fact that Ascomycota fungi are generally easier to cultivate *in vitro* (Addy et al., 2005; Krishnamurthy et al., 2008; Baker et al., 2009). It is worth noting that the Phylum Ascomycota is the largest phylum in the Kingdom Fungi, with approximately 64,000 known species (Moore et al., 2011). Morphologically, Ascomycota is characterized by the production of ascospores within sac-shaped cells called asci (Mueller et al., 2004).

The presence of PKS genes provides support for the potential of endophytic fungi to produce natural bioactive compounds. PKS, which stands for polyketide synthase, is a multifunctional enzyme involved in the biosynthesis of a wide range of polyketide compounds (Hopwood, 1997). Polyketides are important secondary metabolites that are produced by various organisms, with extensive research conducted on their production in bacteria and fungi. Polyketides have been recognized for their diverse range of functions. They have been widely utilized in human medicine as antimicrobial, antifungal, immunosuppressant, and anticancer agents (Rude, 2004). In veterinary medicine, polyketides have been used as antibiotics and anthelmintics. Furthermore, in agriculture, polyketides have shown potential as insecticides and have been investigated for their antimicrobial activity against plant pathogens and microbial competitors (Lee et al., 2001; Rude, 2004).

Additionally, polyketides have been found to exhibit phytotoxic properties, making them potential candidates for agricultural applications (Prado, 2012).

The detection and characterization of fungal polyketides have historically been challenging, limiting the study of these compounds. However, with the availability of fungal genomic sequences, comprehensive analysis of the genetic potential of fungi to produce polyketides has become feasible (Gaffoor et al., 2005). To specifically amplify and screen gene sequences related to polyketide synthesis, highly specific primers are employed. In this study, gene-specific primers called KAF1 and KAR1 were utilized to screen for the presence of the ketoacyl synthase (KS) domain, which is a specific portion of the polyketide synthase (PKS) gene. The KAF1 forward primer was designed based on amino acid sequences within the conserved β-ketoacyl synthase domain of the reducing type fungal domain. On the other hand, the KAR1 reverse primer was designed based on the acyltransferase (AT) domain. By using these specific primers, the study aimed to target and amplify the regions of the PKS gene associated with polyketide synthesis, allowing for the detection and screening of fungal strains for the presence of PKS genes and their potential to produce polyketides.

Among the 16 isolates tested, 15 showed positive results for the presence of PKS genes as indicated by the presence of a band, as illustrated in Figure 4. The amplified DNA fragments obtained were approximately 700bp in length. These positive isolates belonged to three different genera: *Aspergillus*, *Cladosporium*, and *Penicillium*. This finding aligns with a study conducted by Wang et al. (2015), where the presence of the ketoacyl synthase domain was also observed in *Aspergillus* and *Penicillium* species. However, in contrast to our results, the *Cladosporium* isolates in their study did not exhibit the presence of the ketoacyl synthase domain.

Upon sequencing all the PCR products, it was observed that they exhibited low homology to other PKS genes based on BLASTn searches, as indicated in Table 3. This could be due to the limited size of the current molecular database, which may not contain all the fungal gene sequences available (Wang et al., 2015). Interestingly, when constructing the phylogenetic tree, the isolates showed divergence from their closest matches in the NCBI database, suggesting the presence of potentially novel ketoacyl synthase domains within the PKS gene amplified using the KAF1/KAR1 primer pair, as depicted in Fig. 5. The phylogenetic analysis revealed that the KS domain sequences of four amplified PKS sequences formed distinct clades with their corresponding identified genera and closely related PKS sequences listed in Table 3. This also shows a closer evolutionary relationship between fungal species belonging to the same clade. For instance, the isolates S3I4 and S3I1, which belonged to the genus *Penicillium*, formed a clade with the *Penicillium* sp. ketoacyl synthase gene. Similarly, the isolate S3I2, assigned to the genus *Cladosporium*, formed a clade with the *Cladosporium* sp. polyketide synthase gene. These results suggest that the ITS identity possibly corroborates the PKS gene identity. These findings indicate the possibility of novel ketoacyl synthase domain sequences within the isolated strains that have not yet been characterized in the existing literature. It suggests that the molecularly identified isolates may possess unique variations in their PKS gene sequences, emphasizing the potential for discovering novel polyketide synthesis pathways and bioactive compounds.

It has been stressed that antagonistic activity to plant pathogens could be attributed to the presence of bioactive compounds synthesized by fungal endophytes (Wang et al., 2015). Therefore, it is vital to study the antagonistic activity against *X. oryzae* pv. *oryzae* of isolated foliar endophytic fungi in this research.

In this study, the antimicrobial properties of the isolated foliar endophytes were analyzed quantitatively. The results, depicted in Figure 6, revealed that four isolates of the isolates exhibited antagonistic activity against *X. oryzae* pv. *oryzae*, as evidenced statistically comparable change in absorbance as the positive control. Notably, the isolates *Aspergillus spelaeus* (SII2), *Penicillium steckii* (S3I1), *Penicillium chrysogenum* (S3I4),

and *Cladosporium oxysporum* (S3I2) displayed significant antagonistic effects against *X. oryzae* pv. *oryzae*. The level of antagonistic activity demonstrated by these isolates was comparable to or even greater than that of Chloramphenicol, a broad-spectrum antibiotic drug commonly used for treating bacterial infections. It is worth noting that Chloramphenicol has previously shown effective inhibition of *X. oryzae* pv. *oryzae* in other studies (Khan et al., 2011).

Recent studies have demonstrated that representatives of the genera *Aspergillus* and *Penicillium* possess PKS genes and exhibit antimicrobial activity against phytopathogens (Wang et al., 2015). Therefore, it is plausible to attribute the observed antimicrobial activity of these fungal endophytes to the presence of PKS genes and the subsequent expression of these genes. In the case of *Cladosporium*, the antimicrobial activity may be linked to the production of secondary metabolites associated with its pathogenicity. *Cladosporium oxysporum* is a well-known generalist fungus and has been identified as a pathogen for grapevines (Brum et al., 2012). The secondary metabolites produced by *Cladosporium* may contribute to its ability to antagonize other microorganisms, including phytopathogens.

The findings of this study hold significant potential in identifying candidates for the screening of bioactive compounds and novel secondary metabolites synthesized by foliar endophytic fungi (Rocha et al., 2011). The observed possible antimicrobial activity against *X. oryzae* pv. *oryzae* suggests that these fungal isolates may harbor bioactive compounds with the potential for use in biological control strategies. However, further research is still needed to fully understand the role of endophytic fungi in the biological control of *X. oryzae* pv. *oryzae*. Additional studies can shed light on the mechanisms by which these fungal endophytes exert their antagonistic effects and explore their potential as biocontrol agents in the context of rice disease management. Furthermore, investigating the role of endophytic fungi in maintaining the microbiota balance of Diket red rice is crucial. Understanding their interactions with other microorganisms present in the rice plant can provide insights into the ecological significance of these fungi and their potential contributions to the overall health and well-being of the rice plant.

5 Conclusions

In summary, this study showed that four culturable endophytic fungi isolated from Diket Red rice (*A. spelaeus, P. steckii, P. chrysogenum* and *C. oxysporum*) have possible *in vitro* antimicrobial activity against *X. oryzae* pv. *oryzae*, and thus, may be a potential biocontrol for BLB. The endophytic fungi exhibiting the presence of PKS genes are possible sources of bioactive polyketides with various applications especially in agriculture. Based on these results, it is recommended that: (1) further analysis may be done on PKS genes using other degenerate primers to establish the identity of these genes; and, (2) perform metabolomic analysis on the ethyl acetate extracts of foliar endophytic fungi isolated from Diket red rice to determine the presence of specific polyketides and/compounds that may have caused the antimicrobial activity observed.

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References

Aban, JL. 2019. Isolation, molecular identification, phylogenetic analysis, and biodiversity of root symbiotic fungi (RSF) from *Drynaria quercifolia* L. IAMURE International Journal of Ecology and Conservation, 27(1): 1-1

Addy HD, Piercey MM, Currah RS. 2005. Microfungal endophytes in roots. Canadian Journal of Botany, 83: 1-13

Amnuaykanjanasin A, Punya J, Paungmoung P, Rungrod A, Tachaleat A, Pongpattanakitshote S, Tanticharoen M. 2005. Diversity of type I polyketide synthase genes in the wood-decay fungus *Xylaria sp*. BCC 1067. FEMS microbiology letters, 251(1): 125-136

Arnold AE, Lutzoni F. 2007. Diversity and host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots? Ecology. 88(3): 541-549

Baker PW, Kennedy J, Dobson ADW, Marchesi JR. 2009. Phylogenetic diversity and antimicrobial activities of fungi associated with Haliclona simulans isolated from Irish Coastal Waters. Marine Biotechnology, 11: 540-547

Bhatnagar D, Ehrlich KC, Cleveland TE. 2003. Molecular genetic analysis and regulation of aflatoxin biosynthesis. Applied Microbiology and Biotechnology, 61(2): 83-93

Borchardt JK. 1999. Combinatorial biosynthesis: panning for pharmaceutical gold. Modern Drug Discovery. 2: 22-29

Brum MCP, Araújo, WL, Maki, CS, Azevedo JL. 2012. Endophytic fungi from *Vitis labrusca* L. ('Niagara Rosada') and its potential for the biological control *of Fusarium oxysporum*. Genetics and Molecular Research, 11: 4187-4197

Cannon, PA, Kirk, P. 2007. Fungal Families of the World. Kyodo Press, Singapore

Challis GL, Hopwood DA. 2003. Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* species. Proceedings of the National Academy of Sciences, 100(Suppl 2): 14555-14561

Gaffoor I, Brown DW, Plattner R, Proctor RH, Qi W, Trail F. 2005. Functional analysis of the polyketide synthase genes in the filamentous fungus *Gibberella zeae* (anamorph *Fusarium graminearum*). Eukaryotic Cell. 4(11): 1926-1933. https://doi.org/10.1128/EC.4.11.1926-1933.2005

Higginbotham SJ, Arnold AE, Ibañez A, Spadafora C, Coley PD, Kursar TA. 2013. Bioactivity of fungal endophytes as a function of endophyte taxonomy and the taxonomy and distribution of their host plants. PLoS ONE. 8(9): e73192. https://doi.org/10.1371/journal.pone.0073192

Hipol RM. 2014. Antioxidant potentials of culturable endophytic yeasts from *Phragmites australis* Cav. (Trin) ex Steud. from copper-contaminated mining site in Mankayan, Benguet. PhD dissertation. University of the Philippines Los Baños, Laguna, Philippines

Hopwood DA. 1997. Genetic contributions to understanding polyketide synthases. Chemical Reviews, 97(7): 2465-2498

International Rice Research Institute. 2003. Rice in the Philippines. Retrieved October 13, 2015 from irri.org/our-work/locations/philippines

Khan AL, Hamayun M, Kim YH, Kang SM, Lee IJ. 2011. Ameliorative symbiosis of endophyte (*Penicillium funiculosum* LHL06) under salt stress elevated plant growth of *Glycine max* L. Plant Physiology and Biochemistry, 49(8): 852-861

Khush GS. 2005. What it will take to feed 5.0 billion rice consumers in 2030. Plant Molecular Biology, 59(1): 1-6

Krishnamurthy YL, Naik SB, Jayaram S. 2008. Fungal communities in herbaceous medicinal plants from the Malnad region, Southern India. Microbes and Environments 23: 24-28

Kusari P, Kusari S, Spiteller M, Kayser O. 2013. Endophytic fungi harbored in *Cannabis sativa* L.: diversity and potential as biocontrol agents against host plant-specific phytopathogens. Fungal Diversity, 60(1): 137-151

Kusari S, Singh, S, Jayabaskaran C. 2014. Biotechnological potential of plant-associated endophytic fungi: hope versus hype. Trends in Biotechnology, 32(6): 297-303

Lee T, Yun SH, Hodge K, Humber R, Krasnoff S, Turgeon G., Yoder O, Gibson D. 2001. Polyketide synthase genes in insect-and nematode-associated fungi. Applied Microbiology and Biotechnology, 56(1-2): 181-187. https://doi.org/10.1007/s002530100637

Liu XH, Miao FP, Li XD, Yin XL, JI Ny. 2012. A new sesquiterpene from an endophytic *Aspergillus versicolor* strain. Natural Products Communications, 7(7): 819-820

Liu X, Wang M, Chen W, Xu B, LI X, Guo W. 2023. Endophytic fungus *Cladosporium* sp. LC18 from maize root exhibits antifungal activity against *Fusarium fujikuroi* and *Fusarium graminearum*. Journal of Microbiology, Biotechnology and Food Sciences, 13(1): 39-47

Matlock BC, Beringer RW. 2011. Analyzing Differences in Bacterial Optical Density Measurements between Spectrophotometers; Technical Note No. 52236. Thermo Fisher Scientific Inc

Mayorga ME, Timberlake WE. 1992. The developmentally regulated *Aspergillus nidulans* wA gene encodes a polypeptide homologous to polyketide and fatty acid synthases. Molecular and General Genetics, 235(2-3): 205-212

Moore D, Robson G, Tricini A. 2011. 21st Century Guidebook to Fungi. TJ International Ltd, Padstow, United Kingdom

Mueller G, Bills F, Foster M. 2004. Biodiversity of Fungi. Elsevier Inc, China

Naik, BS, Shashikala, J, Krishnamurthy, YL. 2006. Study on the diversity of endophytic communities from rice (*Oryza sativa* L.) and their antagonistic activities in vitro. Microbiological Research. Retrieved from http://www.sciencedirect.com/science/article/pii/S0944501307000389

Newman D, Cragg GM. 2007. Natural products as sources of new drugs over the last 25 Years. Journal of Natural Products, 70(3): 461-477

Pablo CHD, Pagaduan JRR, Langres HKC, Hipol RM 2020 – Plant growth-promoting characteristics of root fungal endophytes isolated from a traditional Cordillera rice landrace. Studies in Fungi 5(1): 536-549. doi: 10.5943/sif/5/1/34

Prado S, Li, Nay B. 2012. Diversity and ecological significance of fungal endophyte natural products. Studies in Natural Products Chemistry: Bioactive Natural Products (Part P), 36: 249

Ray D, Mueller ND, West PC, Foley JA. 2019. Climate change threatens global food security. Science, 365(6455): 981-983

Rocha ACS, Garcia D, Uetanabaro APT, Carneiro RTO, Araújo IS, Mattos CRR, GÓES-NETO A. 2011. Foliar endophytic fungi *from Hevea brasiliensis* and their antagonism on *Microcyclus ulei.* Fungal Diversity, 47: 75-84

Rodriguez RJ, White JR JF, Arnold AE, Redman RS. 2009. Fungal endophytes: diversity and functional roles. New Phytologist, 182(2): 314-330

Rude MA, Khosla C. 2004. Engineered biosynthesis of polyketides in heterologous hosts. Chemical Engineering Science, 59(22): 4693-4701

Sanchez-Marquez S, Bills GF, Zabalgogeazco I. 2008. Diversity and structure of fungal endophytic assemblages from two sympatric coastal grasses. Fungal Diversity. 33: 87-100

Shibata S, Morishita E, Takeda T, Sakata K. 1966. The structure of aurofusarin. Tetrahedron Letters, 7(40): 4855-4860

Siam R, Srinives P, Vanavichit A, Phumwisit P, Punyawongratana P. 2016. Evaluation of local Thai rice varieties for resistance to bacterial leaf blight disease and blast disease. The 5th Rice Research and Development Network Regional Technical Workshop, International Network for Improvement of Rice Germplasm (INIGERP), Hanoi, Vietnam

Singh K, Singh, A, Pandey S, Srivastava, SD. 2022. Bacterial leaf blight: An emerging threat to global rice production. Frontiers in Sustainable Food Systems, 6, 836190.

Strobel G, Daisy B, Castillo U, Harper J, Gresham J. 1996. An endophytic fungus from Glycine max (soybean) producing a novel class of antibiotics. Nature, 380(6572): 608-610

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular biology and evolution, mst197

Tad-Awan BA, Sagalia EJD, Tosay MP. 2010. Morphological diversity and indigenous knowledge on rice landraces in Benguet, Philippines: towards sustainable conservation and utilization. Philippine Journal of Crop Science

Taylor JW, Hibbett DS, Misra, JK, Amon, E, Sánchez, IF, Luckow, M, Boekhout T, Batra LF, James TY, Geiser DM, Huhndorf SM, Lodge DJ, Reynolds DR, Pfister DH, Schüssler A, Lumbsch HT. 2017. A multigene phylogeny defines novel subfamily groups within the order Eurotiales. Fungal Biology and Pathogens, 102(1): 1-10

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25: 4876-4882

Urry WH, Wehrmeister, HL, Hodge EB, Hidy PH. 1966. The structure of zearalenone. Tetrahedron Letters, 7(27): 3109-3114

Vera Cruz CM, ONA I, Castilla NP, Opulencia R. 2015. Bacterial Blight. Retrieved from http://www.knowledgebank.irri.org/decision-tools/rice-doctor/rice-doctor-fact-sheets/item/bacterial-blight

Wang Y., Gao BL, Li XX, Zhang ZB, Yan RM, Yang HL, Zhu D. 2015. Phylogenetic diversity of culturable endophytic fungi in Dongxiang wild rice (*Oryza rufipogon* Griff), detection of polyketide synthase gene and their antagonistic activity analysis. Fungal Biology, 119(11): 1032-1045

Wang F, Yang S, Wu Z, Zhao Y, Xu J, Zhou M. 2020. Antagonistic and anti-biofilm activities of *Penicillium oxalicum* isolated from *Stellera chamaejasme* L. against *Xanthomonas oryzae* pv. *oryzae*, the causal agent of bacterial leaf blight in rice. World Journal of Microbiology and Biotechnology, 36(3): 50

Zhao Y, Qian G, Fan J, Yin F, Zhou Y, Liu C, Shen Q, Hu B, Liu F. 2012. Identification and characterization of a novel gene, hshB, in *Xanthomonas oryzae pv. oryzicola* co-regulated by quorum sensing and clp. Phytopathology, 102(3): 252-259. https://doi.org/10.1094/PHYTO-06-11-0169

Zubrick JW. 2001. Extraction and Washing Guide. The Organic Chem Lab Survival Manual (5th Edition). Wiley & Sons Inc, New York, USA